J Appl Biol Chem (2013) 56(2), 109−112
http://dx.doi.org/10.3839/jabc.2013.018

Online ISSN 2234-7941
Print ISSN 1976-0442

Original Article: Environmental Sciences

Metabolic Differentiation of *Saccharomyces cerevisiae* by Ketoconazole Treatment

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Received: 19 February 2013 / Accepted: 20 March 2013 / Published Online: 30 June 2013
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Abstract Azole fungicides are one of the most wide-spread antifungal compounds in agriculture and pharmaceutical applications. Their major mode of action is the inhibition of ergosterol biosynthesis, giving depletion of ergosterol, precursors and abnormal steroids. However, metabolic consequences of such inhibition, other than steroidal metabolitesare not well established. Comprehensive metabolic profiles of *Saccharomyces cerevisiae* has been presented in this study. Wild type yeast was treated either with glucose as control or azole fungicide (ketoconazole). Both polar metabolites and lipids were analyzed with gas chromatography-mass spectrometry. Approximately over 180 metabolites were characterized, among which 18 of them were accumulated or depleted by fungicide treatment. Steroid profile gives the most prominent differences, including the accumulation of lanosterol and the depletion of zymosterol and ergosterol. However, the polar metabolite profile was also highly different in pesticide treatment. The concentration of proline and its precursors, glutamate and ornithine were markedly reduced by ketoconazole. Lysine and glycine level was also decreased while the concentrations of serine and homoserine were increased. The overall metabolic profile indicates that azole fungicide treatment induces the depletion of many polar metabolites, which are important in stress response.

Keywords azole · metabolomics · mode of action · pesticide toxicity · proline · *Saccharomyces cerevisiae*

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Introduction

Azole-type fungicides (e.g., ketoconazole) are widely used in many agricultural and medicinal applications. Their major mode of action is the inhibition of steroid biosynthesis, being one of the most important primary metabolites in fungal cell membrane (Schwinn, 1984; Zarn et al, 2003). The primary target of these fungicides is C-14 demethylase, which catalyze the consecutive demethylation at 14th carbon of lanosterol (Schwinn, 1984). Inhibition of this crucial step usually produces several unusual steroids. Accumulation of precursors of ergosterol has also been observed (Marichal et al., 1999). In addition to the fungicidal effects, some azole fungicides have been considered as endocrine disruptors (Zarn et al., 2003). Steroidal metabolites were the major analytical targets during the researches of ergosterol biosynthesis inhibitor (EBI). However, no details have been studied with other primary metabolites, especially comprehensive profiles of polar primary metabolites.

Recently, several analytical methodologies have been developed to profile numerous metabolites all at once, namely metabolomics. Several toxicological aspects of pesticides were evaluated with these approaches (Allen et al., 2004; Ekman et al., 2006; Viant et al., 2006; Wesolowski et al., 2010).

In this study, metabolic profiles were evaluated with Baker's yeast (*Saccharomyces cerevisiae*) with or without the treatment of ketoconazole, an azole fungicide. Briefly, steroids and polar metabolites were extracted from the cells, derivatized, and analyzed with gas chromatograph-mass spectrometer. The differences between the fungidie-treated and controls were compared.

Materials and Methods

Chemicals. Ketoconazole and reagents were obtained from Sigma-Aldrich Korea (Seoul, Korea). The purityof ketoconazole was over 98%.Yeast extract-potato dextrose broth was from BD Korea (Seoul, Korea). Solvents were HPLC grade or higher.

Culture of yeast. The yeast cell was grown on YPD medium containing 2% (vol/vol) glucose, 2% (wt/vol) Bacto Peptone (Difco Laboratories), and 1% (wt/vol) yeast extract (Difco). Pesticide treatment was performed as follows. Aliquots (50 μL) of ketoconazole solution (1000 ppm, dimethyl sulfoxide, DMSO) or DMSO were added to a 1 day-culture (250 mL) and further cultivated at 27°C for 36 h. Three replicate cultures have been performed.

Extraction of metabolites. After 36 h of culture, the whole media was centrifuged at 1600 rpm for 20 min. After removal of supernatant, the pellet was suspended in cold methanol (30 mL). The suspension was processed with Bead-beater for 5 times, 1 min for each cycle.

After removal of glass bead, cellular debris was removed by centrifugation (15000 rpm, 15 min). Aliquot (1 mL) of supernatant was collected for the instrumental analysis of polar metabolites. For the analysis of steroidal metabolites, cell pellet (100 mg) was hydrolyzed with methanolic KOH (10%, 10 mL) at 80°C for 1 h. After cooling to room temperature, steroids were extracted with hexane (10 mL ×3). The hexane extracts (1 mL) was concentrated and derivatized for the instrumental analysis.

Derivatization of metabolites. Small amount of extracts were concentrated under reduced pressure. The residue was dissolved in pyridine (1 mL) and derivatized as follows; To the solution, 100 μL of methoxylamine hydrochloride solution (1000 ppm, pyridine) was added and heat to 60°C for 2 h. After cooling to room temperature, was added N,N-Bis-trimethylsilyl-trifluoroaacetamide/trimethylsilyl chloride (100 μL, BSTFA-TMCS) and heated to 75°C for 1.5 h.

Instrumental analyses. Steroids and polar metabolites were analyzed with gas chromatograph-mass spectrometer (GC-MS, Shimadzu GC-2010 Plus with GCMS-2010 SE), equipped with Rtx-5MS column (30 m, 0.25 μm film thickness, 0.25 mm i.d.; Resteck, USA). Helium was a carrier gas at a flow rate of 1 mL/min. The column temperature for polar metabolite analyses were programmed as follows; 95°C (10 min) and raised to 295°C at a rate of 2°C/min and held for 20 min. The mass spectra of metabolites were obtained in full scan mode.Steroidal metabolites were analyzed by the following condition; Initial column temperature, 160°C (10 min) and raised to 300°C at a rate of 2.5°C/min and held for 20 min.

Results and Discussion

There were large numbers of metabolites, giving different concentration profiles by the treatment of ketoconazole. The most noticeable difference was observed with steroidal metabolites (Fig. 1). Among the several steroids, accumulation of lansterol was the largest difference (Fig. 1). Several precursors of ergosterol were also observed. Metabolite **8** was tentatively determined as lanstenoic acid derivative. It seemed to be partial oxidation product of lansterol, of which the further oxidation and demethylation was inhibited by ketoconazole. In addition, several minor metabolites were differentially accumulated or depleted by

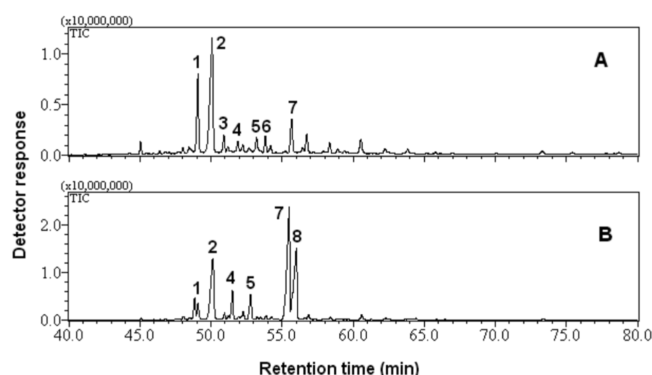

Fig. 1 GC-MS total ion chromatograms of control (A) and ketoconazole (B)-treated yeast cells. Names of metabolites are as follows; 1, zymosterol; 2, ergosterol; 3, 5-dihydroergosterol; 4, fecosterol; 5, 4a-methylzymosterol; 6, 3-Hydroxy-ergosta-8,24(28)-diene; 7, lanosterol; 8, 3a,12a-dihydroxy-24-methylene-lanost-8-en-26-oic acid.

ketoconazole treatment. Those include 5-dihydroergosterol (**3**), fecosterol (**4**), 4a-methylzymosterol (**5**), and 3-Hydroxy-ergosta-8,24(28)-diene (**6**).As documented in many researches, the accumulation of **8** indicates that major mode of action of ketoconazole and its analogues is the inhibition of C-14 demethylation (Fig. 2).

In comparison with steroids, numerous metabolites were observed in polar metabolite fractions. One hundred eighty seven metabolites were tentatively identified from the GC-MS analyses (Table 1). Trehalose (retention time, Rt, 79.5 min) and orthophosphate (Rt, 26.7 min) were the most abundant primary metabolites. Other metabolites (and their Rts, min), found in high quantities were as follows; alanine (16.4), valine (23.4), glycerol (26.8), isoleucine (27.9), proline (28.2), succinic acid (29.2), serine (31.5), threonine (32.8), malic acid (32.8), pyroglutamate (39.5), glutamic acid (43.7), lysine (47.3), α-glycerophosphate (49.3), citric acid (51.4), inositol (60.9), glucose-6-phosphate (64.7), and sucrose (79.1 min). Among the abundant metabolites, some metabolites were differentially accumulated in control or ketoconazole-treated cells (Table 2). For example, several amino acids (glycine, isoleucine, proline, lysine, pyroglutamate, and cysteine) were depleted by ketoconazole-treatment while accumulations of some amino acids (serine, homoserine, and aspartate) were observed by the similar treatment. A metabolite (Rt, 36.39 min) was tentatively identified as aminovalerolactam, of which may be a lactone product of ornithine. In addition to the major metabolites, several minor metabolites also gave differential accumulation patterns. The concentration of uracil was decreased approximately 3 fold in ketoconazole-treated cells. It is noteworthy that uridine level was also decreased by the fungicide treatment. In comparison with the above mentioned metabolites, concentrations of some amino acid (histidine, tyrosine, and tryptophan) and several carbohydrates (e.g., glucose, fructose, trehalose) did not change. In overall, ketoconazole induces large metabolic differences both of steroids and polar metabolites.

Recent study with *Candida albicans* indicates that a number of

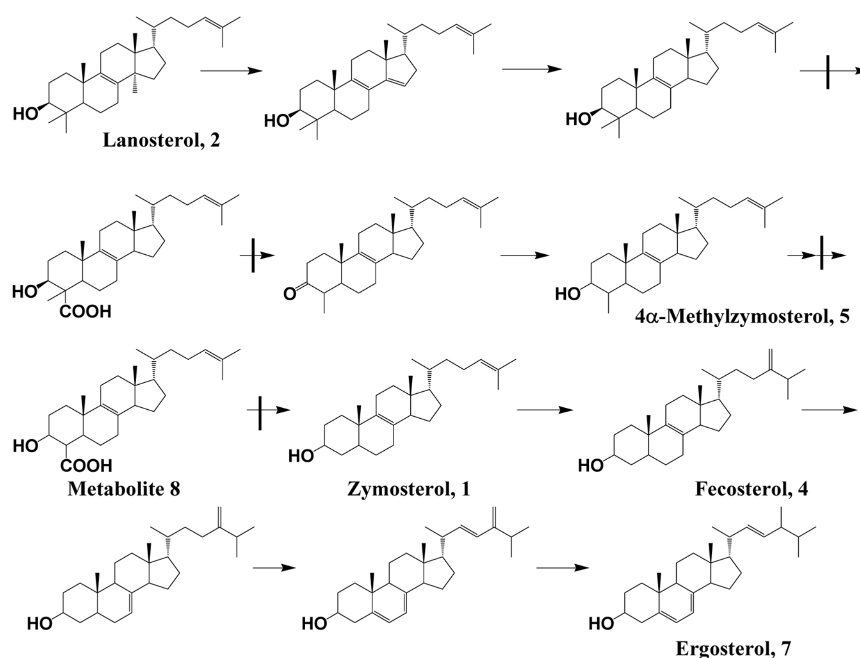

Fig. 2 Biosynthetic pathways of ergosterol and inhibition by ketoconazole. Inhibitory action of ketoconazole was described as thick bars.

transcriptomes have been changed by the treatment of azole fungicide (Liu et al., 2005). In addition to those related to the ergosterol biosynthesis, azole fungicide-treatment induces up-/ down regulation of many othergenes, including metabolic enzymes of amino acid, carbohydrate, lipids, cellular stress response (Liu et al., 2005; Sorgo et al., 2011). Although large change of cellular surface polarity and ultrastructure have been reported by azole fungicide-treatment (Zarn et al., 2003; Sorgo et al., 2011), leakage of intracellular metabolites were barely observed in *C. albicans* (Sorgo et al., 2011). The biological role of ergosterol is rather related to the maintenance of functional integrity of membrane proteins (Iwaki et al., 2008). Many cell membrane protein proteins may affected by the depletion of ergosterol (e.g., transporters). Cellular organisms, including fungi usually give similar metabolic response to chemical stressors. For example, the levels of some metabolites (e.g., citrate, glucose, lactate, succinate etc.) are commonly up- or down-regulated during the chemical stress (Robertson, 2005). Oxidative stress-related response is another common phenomenon in microbial cells under toxic chemical treatment (Tremaroli et al., 2009). Differential metabolism of amino acid is one of the most representative examples, including branched amino acid (valine, leucine, and isoleucine) and glycine. Interestingly, proline concentration was markedly reduced by ketoconazole treatment. Recently, proline has been reported as stress protectant in some plant and microorganism (Hare and Cress, 1997; Hiroshi, 2008; Kaino and Takagi, 2008). Among several biochemical rolls, proline has been reported as osmoregulator in yeast (Hiroshi, 2008). These results suggested that ketoconazole-treatment may induce harmful effects on osmotic regulation. Major biosynthesis of prolineisthrough glutamate and ornithine (Kishor et al., 2005). In ketoconazole-treated culture, the concentration of both metabolites were markedly reduced (Table

Table 1 List of polar metabolites from the yeast cells

Class	# of metabolites	Representative metabolites
Aldehyde/alkanol	6	Glycerol, 1,2- and 1,3-propanediol
Amines	2	Phenethylamine, tryptamine
Amino acids	41	20 essential amino acids, aminolevulinic acid, ornithine, homoserine, homocysteine, N-Acetylglycine, pyroglutamates, aminovlaerolactam
Amino alcohol	2	Ethanolamine
Carbohydrates	32	Hexose (glucose, fructose), pentose (ribose), phosphates of hexose and pentose
Fatty acid/lipid	19	Hexadecanoic acid, Oleic acid, octadecanoic acid, Eicosanoic acid
Nucleotides	12	Adenine, uracil, thymine, AMP, UMP, TMP
Organic acid	29	Glyceric acid, succinic acid, malonic acid, pyruvic acid, furmarc acid, citric acid, citramalic acid
Sugar alcohol	11	Inositol, inositol monophosphate, miscellaneous hexitol
Miscellaneous	33	Nicotinic acid, panthothenol, nicotinamide, picolinic acid

Table 2 Metabolites, accumulated or depleted in ketoconazole-treated yeast cells

ID	Name	Retention Time (Rt, min)	Percent differences*
1	Glycine	17.69	-243±47
2	Pyruvic acid	18.82	178±42
3	Isoleucine	27.97	-68±12
4	Proline	28.20	-47±18
5	Serine	31.58	185±27
6	Homoserine	36.05	421±43
7	Diaminovalerolactone	36.39	-245±31
8	4-Aminobutyric acid	38.71	-47±8
9	Aspartic acid	39.37	172±37
10	Pyroglutamate	39.51	182±46
11	Cysteine	40.27	-58±10
12	Uracil	41.45	-278±15
13	Glutamic acid	43.77	-175±29
14	Lysine	47.10	-418±13
15	α-Glycerophosphate	49.29	-198±25
16	Glucose-6-phosphate	64.71	-176±31
17	Uridine	71.87	-48±17
18	Sucrose	78.47	-342±45

*Differences were calculated as follows; (peak areas in ketoconazole-treated sample − control)/control. Average of triplicate experiments ± standard deviation.

2).

In addition, lysine and glycine concentrations were also markedly reduced by the treatment of ketoconazole-treatment. Both amino acids are very important precursors of many biomolecules, including proteins and osmoregulators (e.g., betaines). It is not clear whether the depletion of these metabolites is from the reduced biosynthesis or the enhanced catabolism. Recent study with yeast cells, treated with cadmium have shown that glycine level was rapidly decreased in cadmium-treated yeast while the concentrations of serine and homoserine were increased (Tanaka et al., 2007). Similar response has also been found in this study (Table 2). However, notable difference was also observed. For example, sulfur-containing amino acid (cysteine) was accumulated in cadmium-treated cell, while notable decrease was observed in this study (Table 2).

Another interesting metabolic change by ketoconazole was the decrease of α-glycerophosphate, which is an important precursor of glycerophospholipid biosynthesis. The result indicates that ketoconazole-treatment may also change the cell membrane constituents, other than steroids.

In overall, ketoconazole induces large extent of metabolic differentiation from control experiments. Among over 180 metabolites, several amino acids, organic acids and precursors - approximately 18 have shown marked difference by the treatment of ketoconazole. These results indicate that abnormal metabolism of ergosterol by azole fungicide may also induce large changes of polar metabolite profiles.

Acknowledgments This work was supported by the faculty research fund of Konkuk University in 2010.

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